

# Immunopathogenesis of Experimental Ulcerative Colitis Is Mediated by Eosinophil Peroxidase<sup>1</sup>

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The precise role that individual inflammatory cells and mediators play in the development of gastrointestinal (GI) dysfunction and extraintestinal clinical manifestations of ulcerative colitis (UC) is unknown. In this study, we have used a mouse model of UC to establish a central role for eotaxin and, in turn, eosinophils in the development of the immunopathogenesis of this disease. In this model the administration of dextran sodium sulfate (DSS) induces a prominent colonic eosinophilic inflammation and GI dysfunction (diarrhea with blood and shortening of the colon) that resembles UC in patients. GI dysfunction was associated with evidence of eosinophilic cytolytic degranulation and the release of eosinophil peroxidase (EPO) into the colon lumen. By using IL-5 or eotaxin-deficient mice, we show an important role for eotaxin in eosinophil recruitment into the colon during experimental UC. Furthermore, using EPO-deficient mice and an EPO inhibitor resorcinol we demonstrate that eosinophil-derived peroxidase is critical in the development of GI dysfunction in experimental UC. These findings provide direct evidence of a central role for eosinophils and EPO in GI dysfunction and potentially the immunopathogenesis of UC. *The Journal of Immunology*, 2004, 172: 5664–5675.

Inflammatory bowel diseases (IBD),<sup>3</sup> Crohn's disease, and ulcerative colitis (UC) are chronic, relapsing, remitting gastrointestinal (GI) diseases characterized by chronic inflammation of the intestine (1–3). UC and Crohn's disease are associated with intestinal and extraintestinal clinical manifestations of disease, which include weight loss, diarrhea accompanied by blood and/or mucus, fever, gastric dysmotility, and shortening of the colon (4, 5). UC is a condition that primarily affects the superficial layer of the colon mucosa, and histological analysis showed ulceration of the mucosa, blunting and loss of crypts, and an inflammatory infiltrate (1). The cellular composition of the inflammatory infiltrate in the colon is characterized by increased numbers of CD4<sup>+</sup> T lymphocytes, mast cells, neutrophils, and eosinophils (1, 2).

Recently, there has been increasing interest in the involvement of eosinophils in the pathogenesis of UC (6). Elevated levels of eosinophils have been observed in colonic biopsy samples from UC patients and increased numbers of this cell have been shown to correlate with morphological changes to the GI tract, disease severity, and gastrointestinal dysfunction (7–10). A number of inflammatory mediators (platelet-activating factor, IL-5, and chemokines

(RANTES, macrophage chemoattractant protein, macrophage inhibitory protein, eotaxin-1 (eotaxin), -2, and 3) have chemotactic activity for human eosinophils and are candidates for the control of colonic eosinophilia in UC (11, 12). In particular, IL-5 regulates eosinophil growth, differentiation, and activation and the chemokine eotaxin is primarily involved in the regulation of eosinophil chemotaxis and effector functions including respiratory burst (13).

Eosinophils are multifunctional leukocytes possessing the capacity to initiate or potentiate inflammatory reactions through the release of a range of inflammatory cytokines, chemokines, and lipid mediators (14–16). In addition, eosinophils may induce GI dysfunction through the release of lipid mediators (platelet-activating factor and leukotriene C<sub>4</sub>) and eosinophilic granular proteins (major basic protein (MBP), eosinophil peroxidase (EPO), and eosinophil-associated ribonucleases, i.e., eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN)). Clinical investigations of bowel biopsy specimens from UC patients have demonstrated a correlation between the eosinophil numbers in the mucosa, the levels of MBP, EPO, ECP, and EDN in perfusion fluid samples, and disease severity (6, 8–10). However, the pathological role of the eosinophil and these factors in UC is not understood.

Several experimental models of UC have been developed in mice to dissect out the key cellular and molecular mechanisms predisposing to disease (1, 2, 17). These models mimic certain pathophysiological features of human UC including weight loss, diarrhea with blood and/or mucus, shortening of the colon, crypt abnormalities, and infiltration of inflammatory cells, particularly eosinophils, into the GI mucosa (1, 2, 17). However, these investigations have provided conflicting evidence concerning the contribution of eosinophils in the development of GI dysfunction in experimental UC (1, 2, 17).

In this study we have dissected out the potential contribution of eosinophils to the pathogenesis of UC by using a model of disease that is induced by dextran sulfate sodium (DSS). We show in this model that the administration of DSS induces a prominent colonic eosinophilic inflammation and GI dysfunction (diarrhea with blood and shortening of the colon) which resembles UC in patients. In

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<sup>3</sup> Abbreviations used in this paper: IBD, inflammatory bowel disease; GI, gastrointestinal; EPO, eosinophil peroxidase; DAI, disease activity index; DSS, dextran sodium sulfate; MBP, major basic protein; OPD, o-phenylenediamine; UC, ulcerative colitis; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; HPF, high-power field; WT, wild type; MPO, myeloperoxidase; RNS, reactive nitrogen species; ROM, reactive oxygen metabolite.

addition, studies with IL-5 and eotaxin-deficient mice demonstrate a central role for eosinophils to the development of disease pathogenesis. Significantly, our data demonstrate that GI dysfunction is associated with evidence of eosinophilic cytolytic degranulation and identifies EPO as a key mediator of disease in experimental UC.

## Materials and Methods

### Animals

We used IL-5-deficient (IL-5<sup>-/-</sup>) mice on the C57BL/6 background (18) and mice deficient in eotaxin (eotaxin<sup>-/-</sup>) (19) that were backcrossed 10 generations into the C57BL/6 and their appropriate C57BL/6 wild-type (WT) controls. We also used MBP<sup>-/-</sup> (20), EPO<sup>-/-</sup> (21) on the 129/Ola/Hsd × 129/SvJ background, and strain and aged-matched WT control mice. All mice were obtained from specific pathogen-free facilities at the Australian National University and housed in approved containment facilities. Mice were treated according to the Australian National University animal welfare guidelines and age- and sex-matched animals were used throughout these studies.

### Induction of experimental UC

DSS used for the induction of experimental UC (ICN Biomedical, Costa Mesa, CA) was supplied as the sodium salt with an average  $M_r$  of 41. Drinking water was supplemented with DSS 2.5% (w/v) for 8 days.

### Disease activity index (DAI)

DAI was derived by scoring three major clinical signs (weight loss, diarrhea, and rectal bleeding) (22). The clinical features were scored separately and then correlated with a histological score: DAI = (body weight loss) + (diarrhea score) + (rectal bleeding score).

### Body weight

Changes in body weight were calculated as the difference between the expected and actual weight on a particular day. The formula for predicted body weight was derived by simple regression using the body weight data for the control group. The following formula was used:  $Y = a + kx$ , where  $Y$  = body weight change (loss or gain),  $k$  = daily increase in body weight,  $x$  = day, and  $a$  = starting body weight.

### Diarrhea

The appearance of diarrhea was defined as mucus/fecal material adherent to anal fur. The presence or absence of diarrhea was scored as either 1 or 0, respectively. The presence or absence of diarrhea was confirmed by examination of the colon following completion of the experiment (22). Mice were sacrificed and the colon excised from the animal. Diarrhea was defined by the absence of fecal pellet formation in the colon and the presence of continuous fluid fecal material in the colon.

### Rectal bleeding

The appearance of rectal bleeding was defined as diarrhea containing visible blood and/or mucus or gross rectal bleeding and scored as described for diarrhea.

### Assessment of Inflammation

Assessment of body weight, evaluation of stool consistency (diarrhea), and rectal bleeding were performed on a daily basis. Body weight was expressed as percent body weight change from baseline. Diarrhea and rectal bleeding were defined as described above. The presence/absence of diarrhea and rectal bleeding was given a score of 0 or 1 and the diarrhea/rectal bleeding score (0–2) is the accumulation of these two values.

### Histopathological examination

Animals were sacrificed on day 8 and the colon was excised. The length of the colon was measured using digimatic calipers (Mitutoyo, Kawasaki, Japan). Tissue specimens were then fixed in 4% paraformaldehyde and stained with H&E and Masson's trichrome using standard histological techniques. Percent colon length with mucosal ulceration was determined by performing morphometric analysis of the colon using an ImageProPlus 4.5 software package (Media Cybernetics, Silver Spring, MD). In brief, digital images of longitudinal sections (1–2 cm in length) of H&E-stained colons were produced. Using the ImageProPlus 4.5 software, the length of ulcerated mucosal lining was divided by the total length of the colonic mucosal surface and the value was expressed as a percentage of colon length with mucosal ulceration.

### Detection and quantification of eosinophils by immunohistochemistry

The colon segment of the GI tract was immunostained with antiserum against mouse MBP as previously described (23). Briefly, 5- $\mu$ m sections were quenched with H<sub>2</sub>O<sub>2</sub>, blocked with normal goat serum, and stained with a rabbit anti-murine eosinophil MBP anti-serum as described earlier (23). The slides were then washed and incubated with biotinylated goat anti-rabbit Ab and avidin-peroxidase complex (Vectastain ABC Peroxidase Elite kit; Vector Laboratories, Burlingame, CA). The slides were developed by nickel diaminobenzidine, enhanced cobalt chloride to form a black precipitate, and counterstained with nuclear fast red. Quantification of eosinophils was performed by counting the number of immunoreactive cells from 15 to 25 fields of view (magnification,  $\times 40$ ) from at least four to five random sections per mouse. Values were expressed as eosinophils per high-powered field.

### Lamina propria cell isolation and flow cytometry

Experimental UC was induced as described above. On day 6, control- and DSS-treated mice were sacrificed and the colon was surgically removed. Colonic lamina propria cells were isolated as described in detail in *Current Protocols of Immunology* (24). In brief, 5-cm colon strips were digested in RPMI 1640 supplemented with 10% FCS, 2 mg/ml collagenase (Roche, Basel, Switzerland), 1.2 U/ml Dispase II (Roche), and 5 U/ml DNase I (Pharmacia LKB Biotechnology, Uppsala, Sweden) and incubated at 37°C for 60 min. The mononuclear cells were separated from the epithelial cells by centrifugation at 4°C for 30 min at 1600  $\times$  g in Ficoll-Plaque Plus (Amersham Biosciences, Uppsala, Sweden). The cell suspension was washed and filtered through 40- $\mu$ m mesh. To examine the level of CD4 and intracellular IL-4 and IFN- $\gamma$  expression, lamina propria cells were stained with FITC-conjugated anti-CD4, PE-conjugated rat anti-mouse IFN- $\gamma$ , and Alexa647-conjugated rat anti-mouse IL-4 as described by the manufacturer (BD Pharmingen, San Diego, CA). In brief, the cell suspension was incubated at 10<sup>6</sup> cells/ml with 1  $\mu$ l of GolgiPlug at 4°C for 20 min. The cells were washed with PBS/1% FCS and then incubated with 50  $\mu$ l of 1% FCS/PBS containing FITC-conjugated anti-CD4 (clone GK1.5 BD, 1  $\mu$ g/10<sup>6</sup> cells; BD Pharmingen) for 30 min at 4°C. The cell suspension was washed twice in PBS/1% FCS and resuspended in 100  $\mu$ l of Cytofix/Cytoperm solution and incubated for 20 min at 4°C. The cell suspension was washed twice with 250  $\mu$ l of BDPerm/wash solution and resuspended in 50  $\mu$ l of 1% FCS/PBS containing PE-conjugated rat anti-mouse IFN- $\gamma$  (clone XMGI.2, 0.5  $\mu$ g/10<sup>6</sup> cells) and Alexa647-conjugated rat anti-mouse IL-4 (clone 11B11, 0.5  $\mu$ g/10<sup>6</sup> cells) and incubated for 30 min at 4°C. The cell suspension was again washed twice with 250  $\mu$ l of BDPerm/wash solution and resuspended in 200  $\mu$ l of 1%FCS/PBS for FACS analysis. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences). The ratio of IL-4<sup>+</sup>CD4<sup>+</sup> T cells to IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells was calculated from cell events acquired by using gates set by forward and side scatter and CD4-FITC and IL-4-Alexa647 double-positive cells and CD4-FITC and IFN- $\gamma$ -PE double-positive cells. Negative control samples were incubated with irrelevant isotype-matched Abs in parallel with all experimental samples.

### EPO activity assay

Mice were sacrificed on day 8 and the colon was excised and flushed with 1 ml of PBS solution. The fecal material was vortexed vigorously for 5 min at 4°C and centrifuged at 10,000  $\times$  g for 10 min at 4°C. The supernatant was collected and placed in sterile Eppendorf tubes and stored at -70°C until analysis. EPO activity was measured in the supernatant of cell-free colon flushes as previously described (25). This assay is based on the oxidation of *o*-phenylenediamine (OPD) by EPO in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The EPO substrate solution consisted of 12 mM OPD (Sigma-Aldrich, St. Louis, MO), 0.005% H<sub>2</sub>O<sub>2</sub>, 10 mM HEPES, and 0.22% cetyltrimethylammonium bromide (CTAB). Substrate solution (75  $\mu$ l) was added to cell-free supernatants that were derived from colon flushes (75  $\mu$ l) in a 96-well microplate and incubated at room temperature for 15 min before stopping the reaction with 50  $\mu$ l of cold 8 N sulfuric acid. Absorbance was measured at 490 nm. Standard EPO activity of 100 U/ml was determined as EPO activity produced by 1  $\times$  10<sup>6</sup> purified eosinophils/ $\mu$ l supernatants. Eosinophils were purified from the spleen of CD2-IL-5-transgenic mice as previously described (26).

### Inhibition of EPO activity by resorcinol

Resorcinol (Sigma-Aldrich; concentration range:  $10^{-6}$ – $10^{-13}$  M) was titrated (1/10 serial dilutions) into supernatants isolated from  $2 \times 10^4$  purified eosinophils in a 96-well flat-bottom microplate. Samples (75  $\mu$ l) were added to EPO substrate solution (75  $\mu$ l) and incubated at room temperature for 15 min before stopping the reaction with 50  $\mu$ l of cold 8 N sulfuric acid. Absorbance was measured at 490 nm. EPO activity in the presence of  $10^{-6}$ – $10^{-13}$  M resorcinol was compared with negative control (no resorcinol-diluent) and expressed as percent inhibition of EPO activity.

### Resorcinol treatment in vivo

Mice were provided with drinking water supplemented with 2.5% (w/v) DSS to induce experimental UC and were i.p. injected with 200  $\mu$ l of resorcinol (1.25 mg/kg) or vehicle (PBS/200  $\mu$ l) daily for 8 days. On day 8, mice were sacrificed and disease parameters were analyzed. In some experiments, mice were provided with drinking water supplemented with 2.5% (w/v) DSS for 8 days to induce experimental UC and on days 6 and 7 i.p. injected with 200  $\mu$ l of resorcinol (1.25 mg/kg). The disease score for the latter experiments were derived by the following score: 1 = healthy plus normal stool (formed pellets); 2 = macroscopic bleeding (occult anal bleeding) plus normal stool (formed pellets); 3 = pilar erecti, decreased activity and body mass, macroscopic bleeding (observable anal bleeding), and diarrhea (semiformed stools that do not stick to the anus); 4 = pilar erecti, dehydration, hunched posture, decreased activity and body mass, excreted perianal mucus, macroscopic bleeding (blood around the anus), and diarrhea (pasty and semiformed stools that stick to the anus); and 5 =

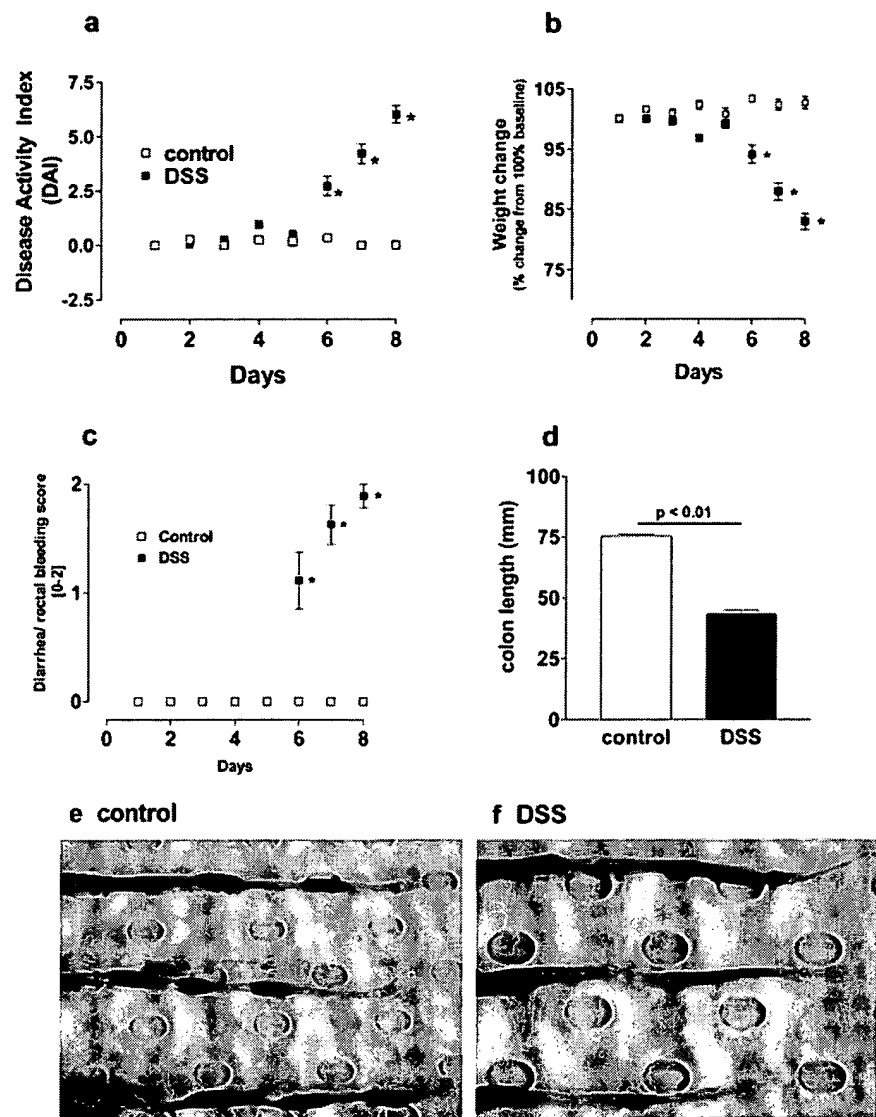
pilar erecti, abdominal distention, shrunken eyes, dehydration, hunched posture, decreased activity and body mass, excreted perianal mucus, gross macroscopic bleeding (blood around the anus or in the cage), and diarrhea (liquid stools that stick to the anus). Since weight variation does not occur within 24–48 h following administration of resorcinol, weight change was not included as a disease parameter.

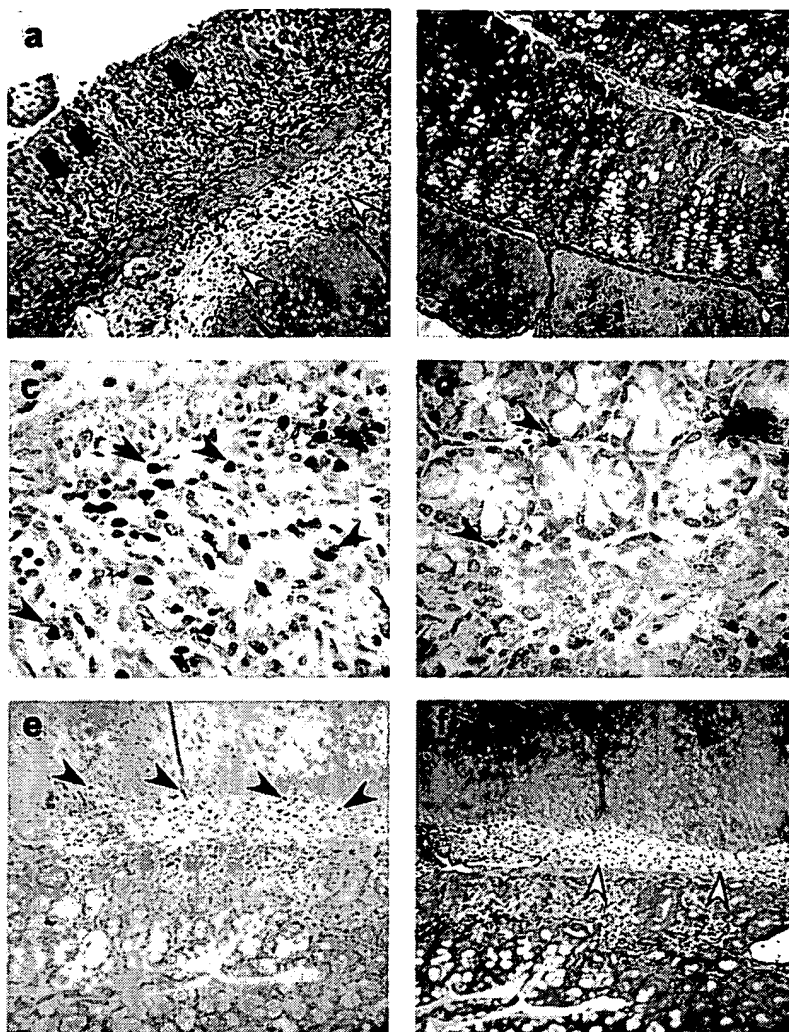
## Results

### DSS-induced experimental UC was associated with eosinophilic inflammation and GI dysfunction

Administration of DSS to C57BL/6 mice induces an acute inflammation of the colon, pronounced weight loss, and bloody diarrhea (Fig. 1a). The first characteristics of pathologies are apparent after 6-day exposure to DSS and weight loss is the most predominant feature of the DAI at this time. Six to 8 days following exposure, mice develop diarrhea, rectal bleeding, and shortening of the colon (Fig. 1, a–f). Histological examination of mouse colons at day 8 showed that DSS-treated mice developed extensive ulceration of the epithelial layer, bowel wall edema, crypt damage, and fibrotic thickening of the muscularis mucosa and dense infiltration of the superficial layers of the mucosa with granulocytes and mononuclear cells (Fig. 2, a–f). We performed immunohistochemistry using a polyclonal antiserum against eosinophil-derived MBP to elucidate whether the

**FIGURE 1.** DSS treatment-induced experimental UC. DAI (a), weight change (b), and diarrhea/rectal bleeding score (c) during the course of DSS treatment in C57BL/6 WT mice. d, Colon lengths (day 8) of control and DSS-treated C57BL/6 WT mice. e, Photograph of control- and (f) DSS-treated colon from C57BL/6 WT mice. The photograph depicts loss of pellet formation in the DSS-treated WT mice, suggesting diarrhea. Data represent the mean  $\pm$  SEM of four to five mice per group. Statistical significance of differences ( $p < 0.05$ ) was determined using Student's unpaired *t* test. Significant differences (\*,  $p < 0.05$ ) between groups. a–c, \*,  $p < 0.01$  as compared with control.





**FIGURE 2.** Histopathology in the colon of DSS-treated mice. *a–f*, Representative photomicrographs of colon from control (*b* and *d*) and DSS-treated (*a*, *c*, *e*, and *f*) C57BL/6 WT mice. *a* and *b*, Photomicrographs of Masson's trichrome stain sections of colon. *c–e*, Immunohistochemically stained sections of colon from control- and DSS-treated mice using the eosinophil-specific anti-MBP Ab. *f*, Masson's trichrome-stained sections of colon from DSS-treated mice. *a*, Arrows depict ulceration of the epithelial cell layer; *a–f*, white filled arrowheads depict fibrotic thickening of the muscularis mucosa; and *c–e*, black filled arrowheads depict MBP-positive eosinophils. Magnification: *a* and *b*,  $\times 100$ ; *c* and *d*,  $\times 500$ ; and *e* and *f*,  $\times 50$ .

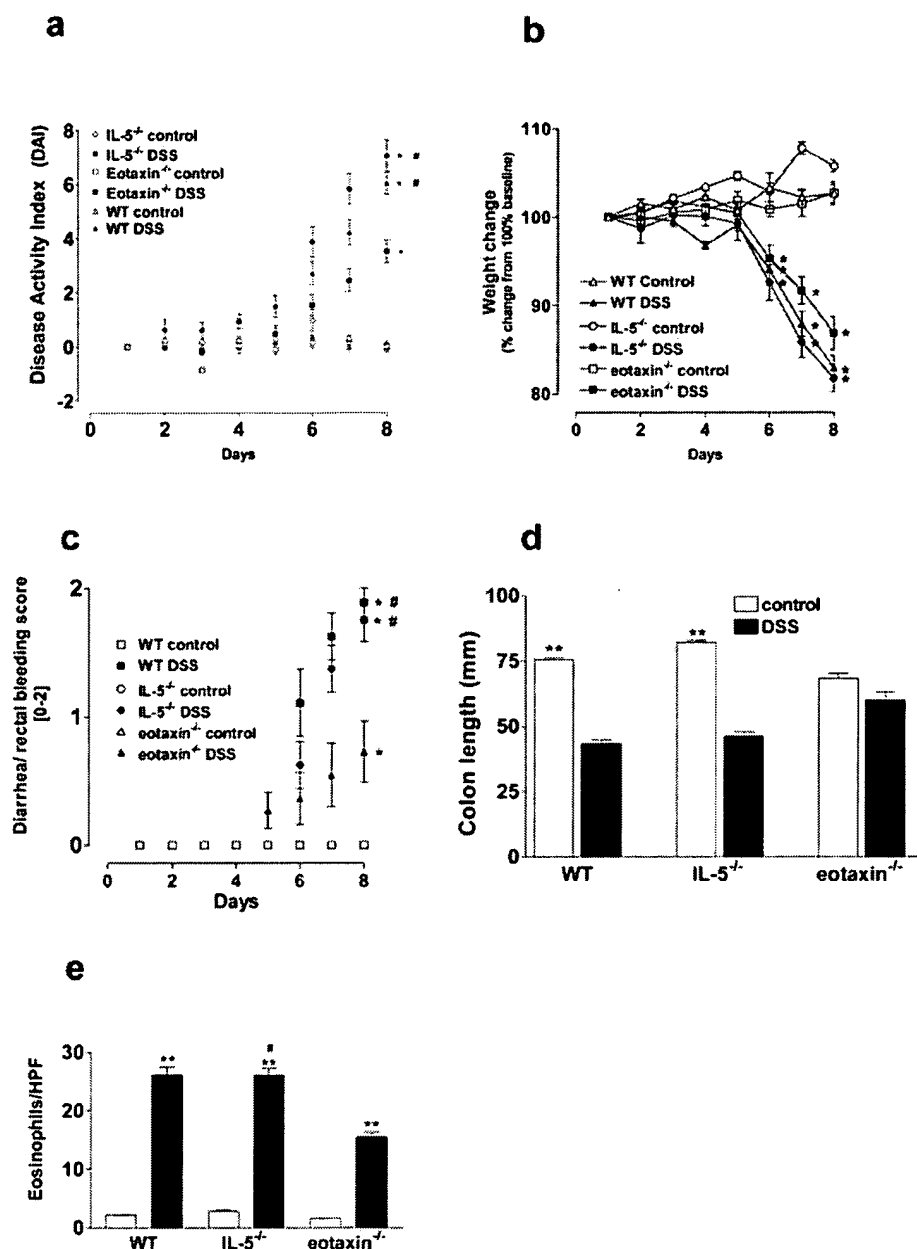
granulocytes were eosinophils. Eosinophils were observed throughout the mucosa and submucosa in DSS-treated mice. In particular, eosinophil numbers were significantly increased in the colon of DSS-treated mice as compared with control-treated mice (eosinophils/high-power field (HPF),  $1.5 \pm 0.05$  vs  $25.9 \pm 1.05$ , mean  $\pm$  SE,  $n = 4$ –5 mice/group, control vs DSS-treated mice, respectively,  $p < 0.005$ ). To characterize the T cell inflammatory response we performed intracellular cytokine staining on colonic lamina propria CD4<sup>+</sup> T cells from control-treated and DSS-treated mice. The ratio of IL-4-positive CD4<sup>+</sup> T cells to IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells in DSS-treated mice was significantly higher than that observed in control mice, suggesting that the T cell inflammatory infiltrate was predominantly Th2 type (ratio of IL-4<sup>+</sup>CD4<sup>+</sup> T cells: IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells;  $0.94 \pm 0.22$  vs  $2.42 \pm 0.53$ , mean  $\pm$  SE,  $n = 3$  mice/group, control vs DSS-treated mice, respectively,  $p < 0.05$ ).

Masson's trichrome stains of colonic sections to evaluate the presence and distribution of collagen showed blue stained, thickly packed collagen fibers, in the mucosa beneath the muscularis mucosa in DSS-treated mice but not in control-treated mice (Fig. 2, *a*, *b*, and *f*). The thick collagen layer present in DSS-treated mice contained large numbers of infiltrating eosinophils (Fig. 2, *e* and *f*). The histopathology of the colon is similar to that observed in UC patients and suggests that a similar pathological mechanism may contribute to tissue damage in both types of inflammation.

#### *Eosinophilic inflammation and GI dysfunction in DSS-induced experimental UC is regulated by eotaxin*

The regulation of eosinophil recruitment during allergic airways disease and trafficking into the upper GI tract (small bowel) at baseline as well as following allergen challenge appears to be regulated by IL-5 and eotaxin (16, 27, 28). We used IL-5- or eotaxin-deficient mice to examine the contribution of IL-5 and eotaxin in eosinophil recruitment into the colon during experimental UC. Neither of the untreated IL-5<sup>-/-</sup> or eotaxin<sup>-/-</sup> mice exhibited any of the pathological symptoms (diarrhea, rectal bleeding, or cachexia) or gross morphological changes to the GI tract normally associated with spontaneous intestinal inflammation. Administration of DSS to IL-5<sup>-/-</sup> mice induced experimental UC, including diarrhea and rectal bleeding, and colon shortening similar to that observed in DSS-treated WT mice (Fig. 3, *a–d*). Drinking volume was similar in all groups (data not shown). Histological examination of mouse colons showed extensive tissue ulceration, massive bowel wall edema, fibrosis of the muscularis mucosa, and a dense cellular infiltration characterized by eosinophils (results not shown). Quantification of eosinophil numbers revealed a significant and near identical increase in the colon of DSS-treated IL-5<sup>-/-</sup> mice as compared with DSS-treated WT mice (Fig. 3*e*). In contrast to WT and IL-5<sup>-/-</sup> mice, DSS-induced experimental UC

**FIGURE 3.** Critical role for eotaxin in DSS-induced experimental UC. DAI (a), weight change (b), and diarrhea/rectal bleeding score (c) during the course of DSS treatment in IL-5<sup>-/-</sup>, eotaxin<sup>-/-</sup>, and strain-matched C57BL/6 WT mice. Colon lengths (d) and eosinophil numbers (e) per HPF in the colon of control and DSS-treated IL-5<sup>-/-</sup>, eotaxin<sup>-/-</sup>, and WT mice. Eosinophils were quantitated by counting 20 similar HPF (magnification,  $\times 40$ ) for each group. Data represent the mean  $\pm$  SEM of four to five random sections per mouse for four to five mice per group. Statistical significance of differences ( $p < 0.05$ ) was determined using Student's unpaired *t* test. Significant differences (\*,  $p < 0.05$ ) between groups. a, \*,  $p < 0.05$  as compared with matched controls; #,  $p < 0.05$  as compared with eotaxin<sup>-/-</sup> DSS. b, \*,  $p < 0.05$  as compared with matched controls. c, \*,  $p < 0.05$  as compared with matched control; #,  $p < 0.05$  as compared with eotaxin<sup>-/-</sup> DSS. d, \*\*,  $p < 0.01$  as compared with matched control. e, #,  $p < 0.05$  as compared with eotaxin<sup>-/-</sup> DSS and \*\*,  $p < 0.01$  as compared with matched control.



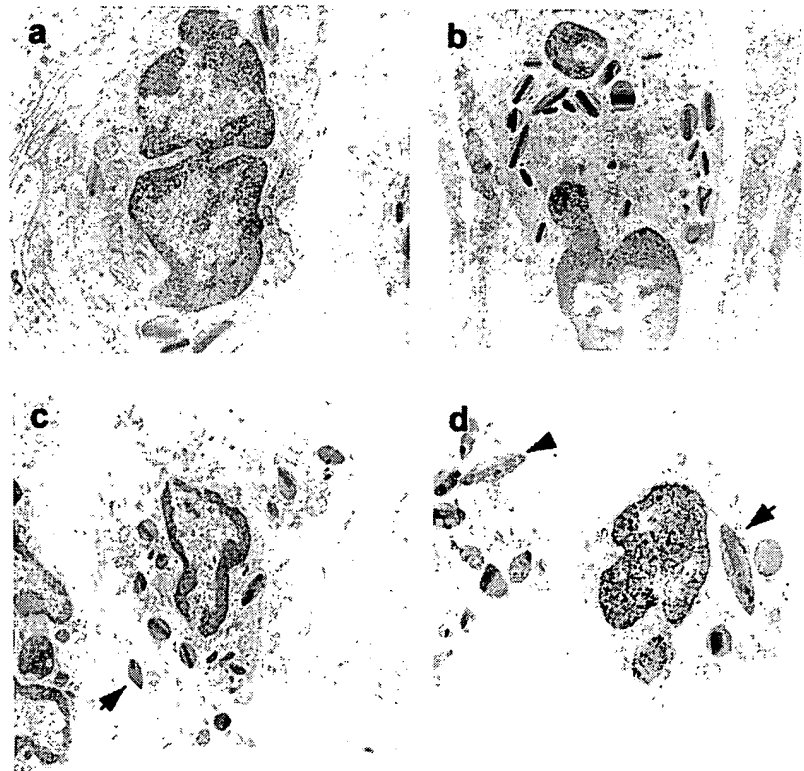
in eotaxin<sup>-/-</sup> mice was significantly attenuated (Fig. 3, a–d). The attenuation of experimental UC in eotaxin<sup>-/-</sup> mice was associated with a reduction (~45% compared with DSS-treated WT and IL-5<sup>-/-</sup> mice) in the number of eosinophils infiltrating into the colon (Fig. 3, a–e).

#### DSS-induced experimental UC is associated with eosinophil cytolytic degranulation

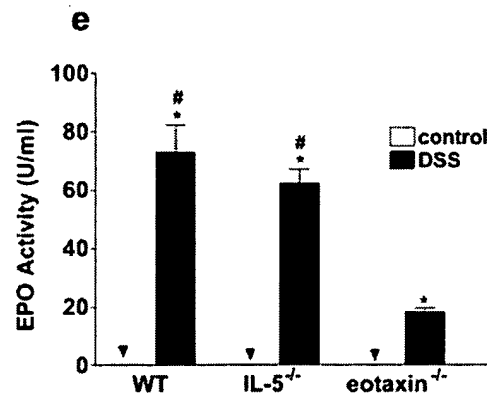
Clinical investigations have provided ultrastructural evidence of eosinophil degranulation in patients with IBD (29–31). In this study, we show administration of DSS induced extensive eosinophilic degranulation. In control-treated mice, colonic eosinophils were primarily located in the lamina propria and possessed features including intact plasma membranes, heterochromatic-segmented nuclei, and abundant granules with characteristic electron-dense cores and matrix (Fig. 4, a and b). In comparison, eosinophils in DSS-treated mice appeared to be undergoing cyto-

lytic eosinophilic degranulation as evidenced by nuclear chromatinolysis, disruption of plasma membrane, and the presence of free eosinophilic granules in the extracellular spaces adjacent to these eosinophils (Fig. 4, c and d).

The demonstration of extensive eosinophilic degranulation suggested that eosinophil granule proteins may contribute to the pathogenesis of experimental UC. We examined the level of EPO in the lumen of the colon of control- and DSS-treated mice and showed that EPO activity in DSS-treated mice was ~1000-fold higher than that observed in control-treated animals (Fig. 4e). We also examined luminal EPO levels in control- and DSS-treated IL-5<sup>-/-</sup> and eotaxin<sup>-/-</sup> mice. In both IL-5<sup>-/-</sup> or eotaxin<sup>-/-</sup> mice luminal EPO levels were elevated in comparison to the control-treated mice (~500- and ~100-fold, respectively). However, consistent with our observation of attenuated experimental UC in eotaxin<sup>-/-</sup> mice, EPO levels and eosinophil intestinal numbers were attenuated when compared with WT and IL-5<sup>-/-</sup> DSS-treated mice (Figs. 3e and 4e).



**FIGURE 4.** DSS treatment induces eosinophil cytolysis and EPO release. *a–d*, Representative electron photomicrographs of eosinophils in the colon of control (*a* and *b*)- and DSS-treated C57BL/6 WT (*c* and *d*) mice. Arrows depict loss of eosinophil plasma membrane and the presence of extracellular protein-laden eosinophilic granules. *e*, Colonic luminal EPO activity in control- and DSS-treated strain-matched C57BL/6 WT, IL-5<sup>-/-</sup>, and eotaxin<sup>-/-</sup> mice. Data represent the mean  $\pm$  SEM of four to five perfusion samples per group. Statistical significance of differences ( $p < 0.05$ ) was determined using Student's unpaired *t* test. *e*, \*,  $p < 0.05$  as compared with matched control; #,  $p < 0.05$  as compared with eotaxin<sup>-/-</sup> DSS.



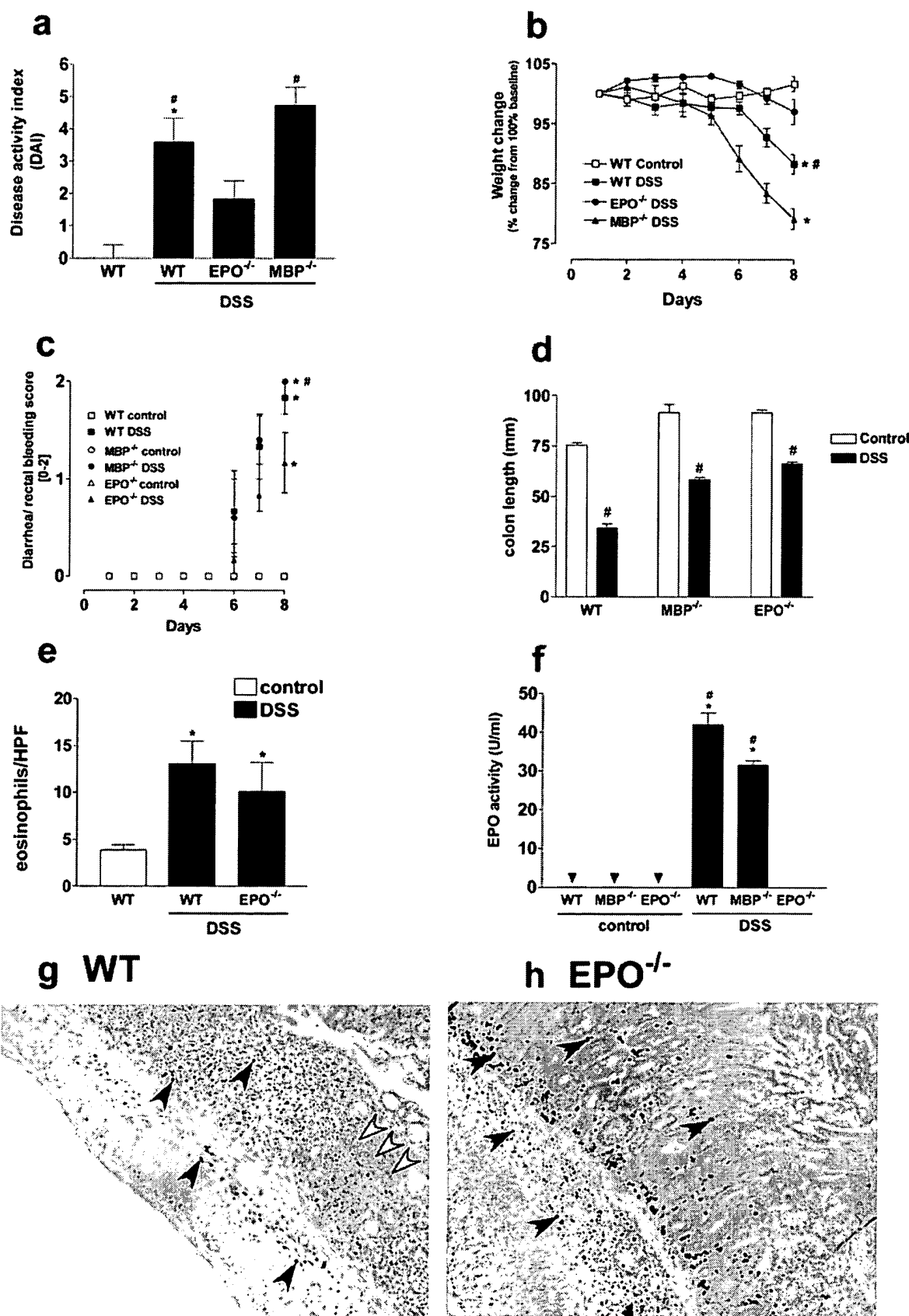
#### *EPO plays an important role in the pathophysiology of DSS-induced experimental UC*

We next challenged MBP<sup>-/-</sup>, EPO<sup>-/-</sup>, and strain-matched WT mice with DSS to examine the contribution of EPO and MBP in DSS-mediated experimental UC. DSS treatment of strain-matched WT mice induced experimental UC similar to that previously described for WT C57BL/6 mice (Fig. 5, *a–d*). Experimental UC was associated with increased DAI, diarrhea, rectal bleeding, eosinophilic inflammation, and colon shortening and elevated levels of colon EPO activity (Fig. 5). Similarly DSS-induced experimental UC in MBP<sup>-/-</sup> mice was comparable to that observed in strain-matched WT mice (Fig. 5, *a–d*). However, in EPO<sup>-/-</sup> mice, DSS-induced colitis was significantly attenuated as compared with either strain-matched WT or MBP<sup>-/-</sup> mice (Fig. 5, *a–d*). This attenuation was associated with the loss of EPO activity in DSS-treated EPO<sup>-/-</sup> mice (Fig. 5f) and occurred despite no reduction in eosinophil intestinal accumulation (Fig. 5e). Histological analysis showed the eosinophils to be dispersed throughout the lamina pro-

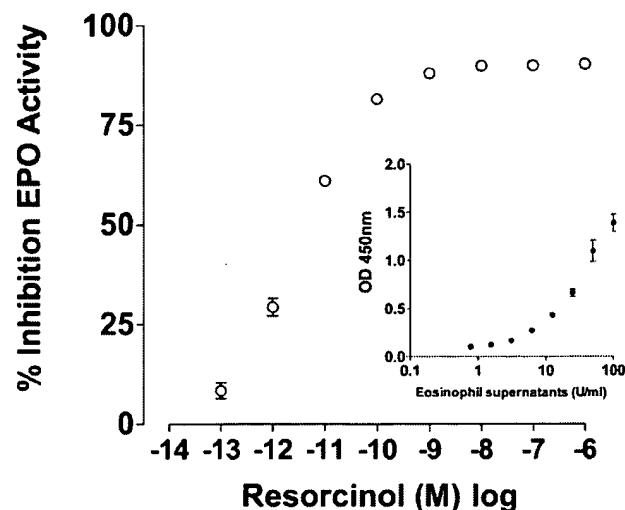
pria and beneath the muscularis mucosa in both DSS-treated WT and EPO<sup>-/-</sup> mice. However, only in the DSS-treated WT mice was the characteristic colonic mucosal ulceration and crypt damage observed (Fig. 5, *g* and *h*). We observed no significant difference in DAI or gross morphological changes to the GI tract between strain-matched WT control-treated and MBP<sup>-/-</sup> and EPO<sup>-/-</sup> control-treated mice (results not shown). Thus, these studies demonstrate that eosinophil-derived EPO plays an important role in the immunopathogenesis of experimental UC.

#### *EPO inhibitor resorcinol can attenuate DSS-induced experimental UC*

The demonstration that EPO plays an important role in the manifestations observed in experimental UC suggests that an inhibitor of EPO activity would attenuate the development of disease. We tested *ex vivo* the suppressive effects of a peroxidase inhibitor resorcinol on EPO activity and found that resorcinol inhibited EPO activity at concentrations as low as 100 fM with an IC<sub>50</sub> of 3 pM

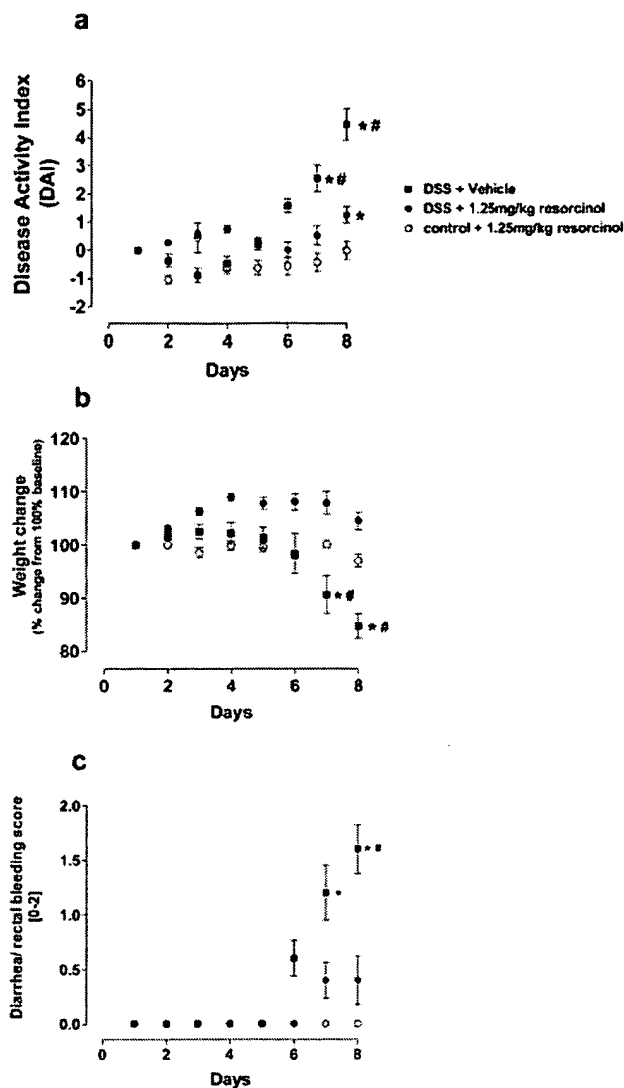


**FIGURE 5.** Critical role for EPO in DSS-induced experimental UC. *a*, DAI on day 8 and weight change (*b*) and diarrhea/rectal bleeding score (*c*) during the course of DSS treatment in strain-matched WT, MBP<sup>-/-</sup>, and EPO<sup>-/-</sup> mice. *d*, Colon lengths and eosinophil numbers per HPF (*e*) in the colon of control and DSS-treated strain-matched WT, MBP<sup>-/-</sup>, and EPO<sup>-/-</sup> mice. *f*, Colonic luminal EPO activity in control and DSS-treated strain-matched WT, MBP<sup>-/-</sup>, and EPO<sup>-/-</sup> mice. No significant difference in the DAI between strain-matched WT control-treated and MBP<sup>-/-</sup> and EPO<sup>-/-</sup> (Figure legend continues)



**FIGURE 6.** Inhibition of EPO activity by resorcinol. Resorcinol (concentration range:  $10^{-6}$ – $10^{-13}$  M) was added with purified eosinophil extracts ( $2 \times 10^5$  purified eosinophils/20 U/ml) in a 96-well flat-bottom microplate. Peroxidase substrate solution (12 mM OPD, 0.005%  $H_2O_2$ , 10 mM HEPES, and 0.22% cetyltrimethylammonium bromide) was added to the extracts and incubated at room temperature for 15 min before stopping the reaction with 50  $\mu$ l of cold 8 N sulfuric acid. Absorbance was measured at 490 nm. Eosinophils were purified from the spleen of CD2-IL-5-transgenic mice as previously described (26). Eosinophils were >99.5% pure. (results not shown). *Inset*, Standard EPO activity assay: EPO activity (100 U/ml) was determined as the amount of EPO activity produced by  $1 \times 10^6$  purified eosinophils/ $\mu$ l extracts. Eosinophil extracts (0.5–100 U/ml) were added to the peroxidase substrate solution and incubated at room temperature for 15 min before stopping the reaction with 50  $\mu$ l of cold 8 N sulfuric acid. Absorbance was measured at 490 nm. Data represent the mean  $\pm$  SEM of triplicate wells.

and maximal inhibition occurred at 10 nM (Fig. 6). Next, we examined the effects of resorcinol on inhibiting EPO activity *in vivo*. Mice were provided with drinking water supplemented with 2.5% (w/v) DSS and were i.p. injected with 200  $\mu$ l of resorcinol (1.25 mg/kg) or vehicle (PBS/200  $\mu$ l) daily for 8 days. Experimental UC in mice treated with resorcinol was significantly attenuated as compared with DSS-treated vehicle-injected mice (Fig. 7, *a–c*). Furthermore, the degree of colon shortening was also reduced in mice treated with DSS and i.p. injected with resorcinol as compared with DSS-treated vehicle-injected mice (Fig. 8*a*). Luminal EPO levels in DSS-treated resorcinol-injected mice were significantly reduced as compared with DSS-treated vehicle-injected mice, although eosinophil levels were equivalent in both groups (Fig. 8, *b* and *c*). Notably, a reduction in pathology following resorcinol treatment was associated with decreased mucosal ulceration of the colon (Fig. 8*d*). To examine whether resorcinol could also suppress established experimental UC, mice received an i.p. injection of vehicle or resorcinol on days 6 and 7 of the 8-day experimental regime (Fig. 9*a*). Experimental UC in mice receiving DSS and administered resorcinol on days 6 and 7 (but not vehicle alone) was significantly attenuated (Fig. 9*a*). Symptoms of experimental UC (rectal bleeding and diarrhea) but not of weight loss

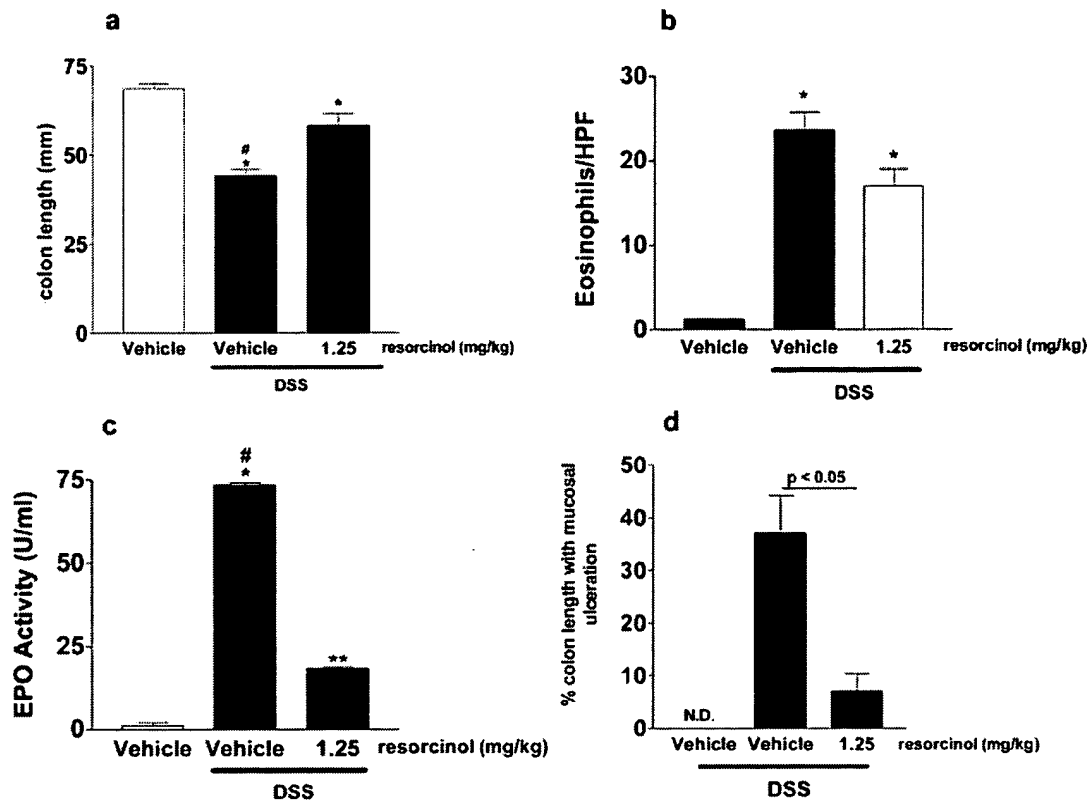


**FIGURE 7.** EPO inhibitor resorcinol attenuates DSS-induced experimental UC. DAI (*a*), weight change (*b*), and diarrhea/rectal bleeding score (*c*) during the course of DSS treatment in vehicle- and 1.25-mg/kg resorcinol-treated C57BL/6 WT mice. Data represent the mean  $\pm$  SEM of four to eight mice per group. *a–c*, \*,  $p < 0.05$  as compared with control plus 1.25-mg/kg resorcinol and #,  $p < 0.01$  as compared with DSS plus 1.25-mg/kg resorcinol.

(results not shown) or colon shortening (colon length:  $39.945 \pm 1.95$  mm vs  $44.30 \pm 2.56$  mm, mean  $\pm$  SE,  $n = 4$ –5 mice/group, vehicle vs resorcinol-treated DSS-challenged mice 24 h following treatment, respectively) were reduced within 24 h and were ablated within 48 h (day 8) of resorcinol treatment (Fig. 9*a*). Notably, the reduction in pathology in DSS-treated resorcinol-injected mice as compared with DSS-treated vehicle-injected mice was not associated with eosinophil recruitment but in fact associated with a reduction in luminal EPO activity. (Fig. 9, *b* and *c*). Collectively,

control-treated mice (results not shown). *g* and *h*, Representative photomicrographs of immunohistochemically stained sections of colon from DSS-treated WT (*g*) and EPO<sup>-/-</sup> (*h*) mice. White filled arrowheads depict mucosal ulceration and black filled arrowheads depict MBP-positive eosinophils. Magnification: *g* and *h*,  $\times 100$ . Data represent the mean  $\pm$  SEM of four to five mice (*a–e*) per group and four to eight (*f*) superfusion samples per group. Statistical significance of differences ( $p < 0.05$ ) was determined using Student's unpaired *t* test. *a*, \*,  $p < 0.05$  as compared with WT control and #,  $p < 0.05$  as compared with EPO<sup>-/-</sup> DSS. *b* and *c*, \*,  $p < 0.05$  as compared with WT control and #,  $p < 0.05$  as compared with EPO<sup>-/-</sup> DSS. *d*, #,  $p < 0.05$  as compared with control. *e*, \*,  $p < 0.05$  as compared with WT control. *f*, \*,  $p < 0.005$  as compared with matched control and #,  $p < 0.01$  as compared with EPO<sup>-/-</sup> DSS.





**FIGURE 8.** EPO inhibitor resorcinol attenuates DSS-induced experimental UC. Colon lengths (*a*) and eosinophil numbers per HPF in the colon (*b*) and of control- and DSS-treated C57BL/6 WT mice administered vehicle or 1.25-mg/kg resorcinol. *c*, Colonic luminal EPO activity in control and DSS-treated C57BL/6 WT mice administered i.p. vehicle or 1.25-mg/kg resorcinol daily. *d*, Percent colon length with mucosal ulceration in control or DSS-treated C57BL/6 WT mice administered i.p. vehicle or 1.25-mg/kg resorcinol daily. *a* and *b*, Data represent the mean  $\pm$  SEM of four to six mice per group and (*c*) four to eight perfusion samples per group. *d*, Data represent the percent colon length with mucosal ulceration  $\pm$  SEM of four to eight mice per group. *a*, \*,  $p < 0.01$  as compared with vehicle; #,  $p < 0.05$  as compared with DSS treated plus 1.25-mg/kg resorcinol. *b*, \*,  $p < 0.05$  as compared with vehicle. *c*, \*,  $p < 0.001$  as compared with vehicle; \*\*,  $p < 0.01$  as compared with vehicle and #,  $p < 0.001$  as compared with DSS treated plus 1.25-mg/kg resorcinol.

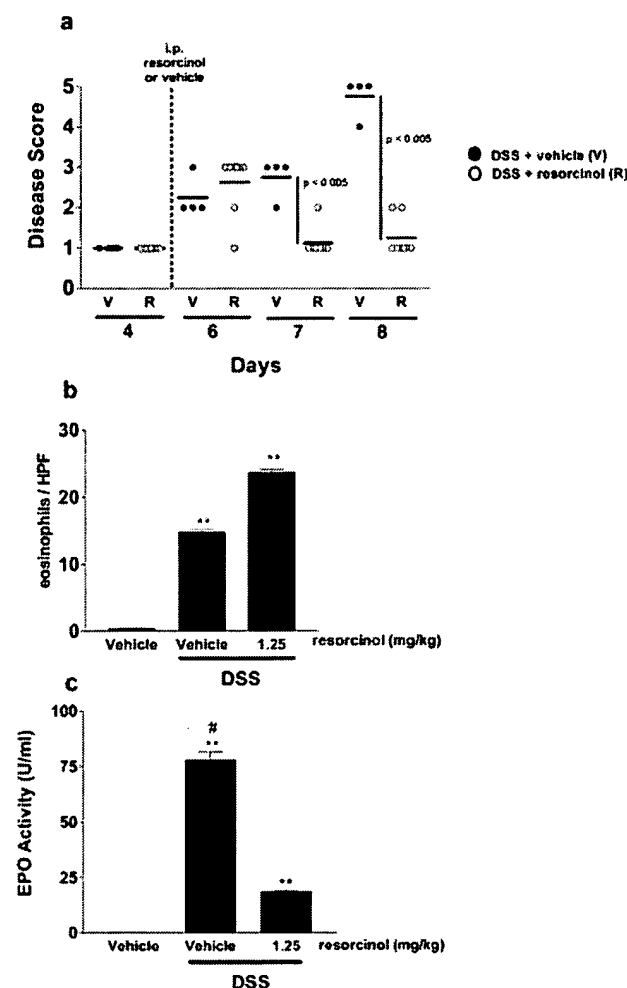
these studies confirm the important role of eosinophil-derived EPO in the pathophysiology of experimental UC and the potential for disease treatment through inhibition of peroxidase activity.

## Discussion

UC is a chronic relapsing inflammatory disease that is primarily driven by an underlying inflammatory response leading to the pathophysiological manifestations of the disease. DSS treatment of mice promotes a chronic experimental UC, which possesses certain pathophysiological features of UC. These features include extensive ulceration of the epithelial layer, massive bowel wall edema, fibrotic thickening of the mucosa, and a dense cellular infiltrate characterized by eosinophils. The similarities between DSS-induced experimental UC and UC suggest that both diseases could be explained by common underlying pathological mechanisms. In this study, we define these mechanisms which include 1) a direct link between eosinophilic inflammation and the pathogenesis of experimental UC; 2) an important role for eotaxin in the recruitment of eosinophils into the colon during experimental UC; 3) that infiltrating eosinophils degranulate by a process of cytolysis releasing protein-laden granules; and 4) that eosinophil-derived EPO is an important mediator in the development of the colitis and that blockade of EPO activity can attenuate the development of experimental UC.

Studies using an oxazolone colitis model, another Th2 colitis model that possesses the immunopathological features of UC, in-

cluding a colonic eosinophilic infiltrate, have demonstrated a critical role for IL-13-producing NK T cells and IL-13 in the pathogenesis of experimental UC (32). During inflammatory responses, IL-13 has been shown to regulate an array of eosinophil-sensitive functions including eotaxin production and eosinophil recruitment and survival (33–36). Elevated levels of eotaxin have also been observed in serum and biopsy samples from patients with UC consistent with a role for eotaxin in eosinophil accumulation into the GI tract during UC (6, 11, 37, 38). Using eotaxin<sup>-/-</sup> mice, we demonstrate an important role for eotaxin in the recruitment of eosinophils into the colon during experimental UC. In light of our studies and previous investigations, it is tempting to speculate that IL-13 derived from NK T cells promotes chemokine production and subsequent eosinophilic infiltration which leads ultimately to disease. Notably, we also demonstrate that eotaxin plays an important role in the expression of the pathophysiological features of experimental UC. Using eotaxin<sup>-/-</sup> mice, we show that while eosinophil levels are reduced by 2-fold, disease pathology (diarrhea, rectal bleeding, and colon shortening) was markedly attenuated. These data suggest that eosinophil recruitment may occur via an eotaxin-independent process; however, eotaxin is critical for the development of the disease pathology. Recently, experimental investigations have demonstrated that eotaxin can induce eosinophil activation and respiratory burst (13). It is possible that eotaxin plays multiple roles in eosinophil function, regulating both trafficking and also eosinophil activation and degranulation and the



**FIGURE 9.** EPO inhibitor resorcinol attenuates DSS-induced experimental UC. *a*, Disease score during the course of DSS treatment in C57BL/6 mice treated with vehicle or 1.25-mg/kg resorcinol on days 6 and 7. Eosinophil numbers per HPF (*b*) and luminal EPO activity in the colon of control- and DSS-treated C57BL/6 WT mice (*c*) administered vehicle or 1.25-mg/kg resorcinol on days 6 and 7. *a*, Data represent individual disease scores per mouse per group. *b* and *c*, Data represent the mean  $\pm$  SEM of four to five mice per group. *b* and *c*, \*\*,  $p < 0.005$  as compared with vehicle; and #,  $p < 0.01$  as compared with 1.25 mg/kg resorcinol.

subsequent development of pathological symptoms. The demonstration of a central role for eotaxin and eosinophils in experimental UC in the present study is in contrast to a previous investigation using IL-5<sup>-/-</sup> mice in a DSS model of colonic injury and inflammation (22). Although we cannot fully explain these inconsistencies, Stevceva et al. (22) used a general histological staining technique (H&E) that poorly differentiates immature and degranulating eosinophils in GI tissues, whereas we used an eosinophil-specific stain (MBP immunohistochemistry) that allows for specific identification and quantification of eosinophils in all activation states.

Previous clinical investigations have demonstrated collagen deposition in the intestinal biopsy samples from patients with IBD (39). The collagen deposition was shown to be associated with cellular inflammation and TGF $\beta$  and insulin-like growth factor I expression (39). One interesting feature of experimental UC that we observed in the present investigation is the extensive deposition of collagen in the colonic submucosa. Notably, eosinophils were interspersed throughout the fibrotic layer, suggesting that eosino-

phils may contribute to collagen deposition. The mechanism causing collagen deposition is currently unknown but it may be related to eosinophil inflammation. TGF $\beta$  is a profibrotic molecule and has been shown to be a potent inducer of collagen deposition (35, 40). Furthermore, recent investigations have demonstrated that eosinophils produce TGF $\beta$  during inflammation (41, 42). The contribution of eosinophil-derived TGF $\beta$  in colonic fibrosis in experimental UC is not yet fully elucidated.

Eosinophils possess an array of cytotoxic granule proteins (MBP, EPO, and eosinophil-associated ribonucleases) that are capable of inducing tissue injury, including epithelial cell damage (14, 43). Eosinophils release these granular proteins and other inflammatory mediators (cytokines and lipid mediators) by a number of mechanisms (granule extrusion (exocytosis), piecemeal degranulation, and cytolysis) (44–47). In the present study, we show that eosinophils undergo cytolytic degranulation, releasing protein-laden eosinophilic granules into the surrounding tissue. Previous experimental and clinical investigations have demonstrated eosinophil cytolysis in gastric ulcer formations and human eosinophilic inflammatory disorders including IBD and atopic dermatitis, suggesting a link between eosinophil degranulation and the development of intestinal pathology (30, 31, 48).

Clinical investigations of bowel biopsy specimens and perfusion fluid samples from UC patients have demonstrated elevated levels of EPO (6, 10). Similarly in experimental UC, colon EPO levels in mice are ~1000-fold higher than those observed in control-treated mice. Using EPO-deficient mice, we demonstrate a critical role for EPO in the pathogenesis of experimental UC. Furthermore, we show that a peroxidase inhibitor resorcinol can also suppress the development of DSS-induced experimental UC. Resorcinol induces irreversible, hydrogen peroxide-dependent loss of peroxidase activity in heme-containing peroxidases (49). This class of peroxidases catalyzes the oxidation of resorcinol to form a reactive radical species that covalently bind to amino acid residues in the distal heme pocket in the enzymes active site, thus blocking function (49, 50). Although resorcinol has been shown to inhibit both myeloperoxidase activity and EPO activity, in vitro analysis suggests that resorcinol is a more potent inhibitor of EPO (25). These findings support the concept that eosinophil-derived EPO is critically linked to manifestations of experimental UC. Although inhibition of EPO did not completely abrogate experimental UC, blockade of EPO as opposed to loss of eotaxin or IL-5 function had a more significant impact on the development of disease. It is noteworthy that clinical investigations have also demonstrated increased levels of a number of other eosinophil granular proteins, including MBP, ECP, and EDN in biopsy samples from UC patients strengthening a causal link to this granulocyte (6, 8–10). We are currently unable to evaluate the role of all of these proteins in experimental UC; however, we did assess the role of MBP in experimental UC and found that this molecule is not critically involved in disease.

Recently studies have suggested an important role for neutrophils in DSS-induced colitis (51–54). Kriegelstein et al. (54) show that DSS treatment promotes a neutrophilic infiltrate and elevated levels of neutrophil-derived myeloperoxidase (MPO) activity in the colon. We observed a minor neutrophil infiltration into the colon following DSS treatment (results not shown); however, the predominant infiltrating cell population was eosinophils. Although we cannot exclude a role for neutrophils and MPO in DSS-induced pathology, we do show that peroxidase activity levels in DSS-treated EPO<sup>-/-</sup> mice is equivalent to those observed in vehicle-treated EPO<sup>-/-</sup> or WT mice, firmly suggesting that EPO, and not

neutrophil derived MPO and/or other peroxidases, primarily contributes to the luminal peroxidase activity and pathology associated with DSS-induced colitis.

EPO catalyzes the oxidation of halides and pseudohalides ( $\text{Cl}^-$ ,  $\text{Br}^-$ , and  $\text{SCN}^-$ ) with the products of respiratory burst ( $\text{O}_2$  and  $\text{H}_2\text{O}_2$ ) to generate cytotoxic oxidants (3-bromotyrosine, 3-chlorotyrosine, and hypothyocynite). These cytotoxic oxidants induce tissue damage and cell death (55). Recently, EPO has also been shown to preferentially catalyze the oxidation of nitrite ( $\text{NO}_2^-$ ), generating the highly toxic reactive nitrogen species (RNS) 3-nitrotyrosine and peroxynitrate (56, 57). Clinical and experimental studies have demonstrated elevated levels of RNS in bronchial aspirates from asthma patients, and that EPO directly contributes to the generation of these reactive nitrogen species (nitrotyrosine and peroxynitrate) (57). UC has also been shown to be associated with increased inducible NO synthase activity as well as NO and RNS production (58). Furthermore, recent clinical studies have demonstrated an imbalance in secondary mucosal antioxidant pathways and production of reactive oxygen metabolites (ROM) including  $\text{H}_2\text{O}_2$  and hypochlorous acid as well as RNS in IBD (59). It is possible that the release of EPO in the lumen during experimental UC leads to the generation of RNS and ROM and the subsequent development of the pathophysiological features of the disease. We are further investigating the role of EPO in the generation of ROM and RNS in experimental UC.

In conclusion, we have shown that during experimental UC, eosinophils transmigrate into the colon where they degranulate, releasing EPO, and induce a progressive colitis resembling UC in patients. Furthermore, blockade of EPO activity by genetic manipulation or by drug treatment suppressed the development of experimental UC and, importantly, administration of an EPO inhibitor reversed established disease. These studies suggest that antagonism of EPO activity may be a therapeutic approach for the treatment of UC.

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